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Autographa californica Multiple Nucleopolyhedrovirus 38K Is a Novel Nucleocapsid Protein That Interacts with VP1054, VP39, VP80, and Itself▽†

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It has been shown that the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *38K* (*ac98*) is required for nucleocapsid assembly. However, the exact role of 38K in nucleocapsid assembly remains unknown. In the present study, we investigated the relationship between 38K and the nucleocapsid. Western blotting using polyclonal antibodies raised against 38K revealed that 38K was expressed in the late phase of infection in AcMNPV-infected *Spodoptera frugiperda* cells and copurified with budded virus (BV) and occlusion-derived virus (ODV). Biochemical fractionation of BV and ODV into the nucleocapsid and envelope components followed by Western blotting showed that 38K was associated with the nucleocapsids. Immunoelectron microscopic analysis revealed that 38K was specifically localized to the nucleocapsids in infected cells and appeared to be distributed over the cylindrical capsid sheath of nucleocapsid. Yeast two-hybrid assays were performed to examine potential interactions between 38K and nine known nucleocapsid shell-associated proteins (PP78/83, PCNA, VP1054, FP25, VLF-1, VP39, BV/ODV-C42, VP80, and P24), three non-nucleocapsid shell-associated proteins (P6.9, PP31, and BV/ODV-E26), and itself. The results revealed that 38K interacted with the nucleocapsid proteins VP1054, VP39, VP80, and 38K itself. These interactions were confirmed by coimmunoprecipitation assays in vivo. These data demonstrate that 38K is a novel nucleocapsid protein and provide a rationale for why 38K is essential for nucleocapsid assembly.

The *Baculoviridae* are a large and diverse family that infects a number of arthropods, mainly members of the order Lepidoptera (39). In the baculovirus life cycle, two types of progeny viruses, the budded virus (BV) and the occlusion-derived virus (ODV), are produced (3). Baculovirus virions consist of rod-shaped nucleocapsids and an envelope (39). The nucleocapsid is composed of three distinguished sections: a base structure on one end, a cylindrical capsid sheath, and a nipple cap on the other end (13). Upon baculovirus infection, an intranuclear viral replication center, designated the virogenic stroma (VS), develops. Viral DNA replication and late gene transcription are carried out in the electron-dense matte of the VS. In contrast, viral genome packaging into the capsid sheaths occurs within the electron-lucent intrastromal spaces (45). Nucleocapsid maturation appears to begin with the assembly of empty capsid sheaths on the base structure. The nucleoprotein core, which is composed of a circular, covalently closed double-stranded DNA genome and the major basic protamine-like core protein P6.9 (46), is concentrated from the base to the apex of the capsid cylinder. The apical cap of the nucleocapsid is oriented toward the matte (13).

The component of the nucleocapsid cap structure has not been well defined. The capsid sheath is formed by stacked ring-like subunits consisting of one major polypeptide, VP39, together with several minor proteins (38). It was proposed that PP78/83, BV/ODV-C42, and ODV-EC27 form a complex at the nucleocapsid base because electron microscopy revealed that PP78/83 is associated with the nucleocapsid base, and BV/ODV-C42 interacts directly with PP78/83 and ODV-EC27 (5, 36, 38). Several additional minor proteins have also been identified to be associated with the nucleocapsid, including PCNA (2), VP1054 (31), FP25 (4, 18, 35, 48), VLF-1 (22, 27, 29, 42, 43, 50), VP80 (21, 25, 30), and P24 (47). PP78/83, VP1054, and VLF-1 have been reported to be required for nucleocapsid assembly (31, 34, 43). Recently, EXON0 (ORF141) (10, 11) and AC142 (26) have also been shown to be nucleocapsid proteins, but neither EXON0 nor AC142 is required for nucleocapsid assembly. EXON0 interacts with the nucleocapsid proteins BV/ODV-C42 and FP25 and is required for the efficient exit of nucleocapsids from the nucleus (10). AC142 is essential for BV production and ODV envelopment (26). Currently, the exact composition of the nucleocapsid and the mechanism by which nucleocapsids are assembled are still unknown.

Mature nucleocapsids have two fates: during the early phase of infection, nucleocapsids move out of the nucleus and obtain an envelope by budding through the plasma membrane to form BVs; in the late phase, nucleocapsids retained in a peristromal compartment, which is called the ring zone, align with de novo membranous profiles end-on and acquire an envelope to form ODVs. ODVs are subsequently incorporated into a paracrys-

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talline matrix consisting mainly of the polyhedrin protein to form polyhedra (45). The compositions of the envelopes of BV and ODV are different from each other to accommodate the respective functions of their two viral forms in the infection cycle (7). When an insect feeds on a plant contaminated with polyhedra, the alkaline midgut fluids dissolve the polyhedra and release infectious virions (ODVs) to initiate the primary infection. BVs produced within the infected midgut epithelial cells are involved in systemic infection from cell to cell (12).

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type species for the family *Baculoviridae* and is the first baculovirus whose genome was completely sequenced (1). AcMNPV *38K* (*ac98*) is a baculovirus core gene, as it is present in all sequenced baculoviruses (16). *38K* was first identified by nucleotide sequence analysis of map units 60.1 to 65.5 of AcMNPV (23), and its 1.1-kb transcript was expressed in the late phase during infection (24). 38K was found to be able to increase early, late, and very late gene expression by the late-expression-factor assay and was able to interact with itself to form homo-oligomers (15). Our previous study revealed that 38K is essential for viral genome packaging into the capsid (49). However, the exact role of 38K in the nucleocapsid assembly process is still unclear.

Recently, flight mass spectrometry showed that the *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) ORF87, a homolog of AcMNPV 38K, was associated with ODV (33), suggesting that 38K is a structural protein. However, with the same strategy, 38K was not identified in the AcMNPV ODV (6) or the *Helicoverpa armigera* nucleopolyhedrovirus ODV (9). It is possible that 38K is present in low abundance and thus not detectable by this method.

In this report, an antiserum against the His-38K fusion protein was raised to detect the expression time course of 38K in AcMNPV-infected Sf9 cells and to determine if 38K is a structural protein. Our results revealed that 38K was expressed in the late phase of infection, as many structural proteins are. By fractionating BV and ODV into the envelope and nucleocapsid, we found that 38K was associated with the nucleocapsids of BV and ODV. Immunoelectron microscopy revealed that 38K specifically localized to the nucleocapsids in infected cells and appeared to be distributed over the cylindrical capsid sheath of the nucleocapsid. Using the yeast two-hybrid and coimmunoprecipitation (co-IP) assays, we were able to detect the interactions of 38K with the nucleocapsid proteins VP39, VP1054, VP80, and 38K itself. Thus, 38K is a nucleocapsid protein. A possible role for this protein in viral nucleocapsid assembly is discussed.

MATERIALS AND METHODS

Viruses, cell lines, and insects. The Sf9 insect cell line, clonal isolate 9 from IPLB-Sf21-AE cells which were derived from the fall armyworm *Spodoptera frugiperda* (44), was cultured at 28°C in TNM-FH medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), penicillin (100 μg/ml), and streptomycin (30 μg/ml). Larvae of *Trichoplusia ni* were reared on an artificial diet at 28°C (19). AcMNPV BVs and ODVs were prepared by infecting third-instar *T. ni* larvae per os with the AcMNPV polyhedra. The titers of BV were determined by a 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay with Sf9 cells as previously described (32). In all virus infection experiments, the virus inoculum was allowed to adsorb for 1 h upon infection at 28°C and was then replaced with fresh medium. Time zero was defined as the time when the virus was inoculated.

Expression of 38K in *Escherichia coli* and production of anti-38K antiserum. Two primers (all primers mentioned in this paper are listed in Table S1 in the supplemental material), 38K5 and 38K3, were designed to amplify the entire *38K* open reading frame (ORF) from the AcMNPV genome. The PCR product was cloned into pMD 18-T (TaKaRa Biotechnology) for sequencing and then subcloned into the expression vector pPROEX HTa (Invitrogen Life Technologies) to generate a recombinant plasmid, pPROEXHTa-38K. In the resulting plasmid, 38K was fused in-frame with a His tag at the C terminus. *E. coli* DH5α cells containing pPROEXHTa-38K were grown at 37°C in LB medium to an optical density at 600 nm of 0.6 and then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested by centrifugation 3 hours later, and the pelleted cells were resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl, pH 7.4) containing lysozyme (Sigma-Aldrich Co.). Cells were incubated for 30 min at room temperature and then lysed by sonication. The pellets were collected by centrifugation at 12,000 rpm for 10 min at 4°C. The inclusion bodies present in the pellets were washed with Triton X-100 and EDTA before being dissolved in inclusion body solubilization buffer (8 M urea, 20 mM Tris, 0.5 M NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol, pH 8.5). His-38K was then purified by chromatography on a Ni-chelating affinity column. Antiserum was prepared by immunizing rabbits with four doses of the purified protein (100 μg protein/dose) with Freund's complete adjuvant. The specificity of the rabbit anti-38K polyclonal antiserum was tested by Western blotting of cells infected or uninfected with AcMNPV or transfected with a *38K* knockout AcMNPV mutant.

Time course analysis of 38K expression. Monolayer Sf9 cells (5×10^6) were infected with wild-type AcMNPV virus at a multiplicity of infection (MOI) of 5 $TCID_{50}$ per cell. At 12, 18, 24, 48, 72, and 96 h postinfection (hpi), cells were collected and centrifuged at 3,000 rpm for 5 min at room temperature. The pelleted cells were washed twice with PBS and resuspended in PBS with an equal volume of 2× protein sample buffer (PSB; 0.25 M Tris-Cl, pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue). Proteins were analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting or stored at −20°C until use.

Purification of BV and ODV. Six hundred third-instar *T. ni* larvae were infected by contamination of the artificial diet with the AcMNPV E2 polyhedra. To purify BV, 15 ml of hemolymph was extracted from the infected insects at 3 days postinfection and clarified twice by centrifugation at 3,000 rpm for 10 min at room temperature, and the supernatant was then centrifuged at 35,000 rpm (Beckman SW41 rotor) for 45 min at 4°C to pellet the virus. The pellets were resuspended in 0.5 ml of 0.1× TE (10 mM Tris, pH 7.4, and 1.0 mM EDTA) containing 5 mM phenylthiocarbamide to inhibit prophenoloxidase activity and then overlaid onto a 15-ml 25% to 56% (wt/vol) continuous sucrose gradient. Gradients were centrifuged at 24,000 rpm (Beckman SW41 rotor) for 90 min at 4°C. The virus band was removed, 1:4 diluted, and repelleted by centrifugation at 24,000 rpm (Beckman SW41 rotor) for 90 min at 4°C. The BV was resuspended in 0.2 ml of 0.1× TE and stored at −20°C.

Polyhedra were isolated from infected insects and purified as previously described (7). The concentration of the purified polyhedra was measured with a counting chamber. ODV was purified according to the method of Braunagel and Summers (7), with modifications. Briefly, 2×10^9 polyhedra were incubated in polyhedra lysis buffer (0.1 M Na_2CO_3, 0.166 M NaCl, and 0.01 M EDTA, pH 10.5) at 37°C for 15 min until the solution became clear, and the suspension was neutralized by adding a 1/10 volume of 0.5 M Tris-HCl (pH 7.5). The ODV suspension was centrifuged at 3,000 rpm for 5 min to remove undissolved polyhedra and other debris, and then the supernatant was layered onto a 15-ml 25% to 56% (wt/vol) continuous sucrose gradient and centrifuged at 18,000 rpm (Beckman SW41 rotor) for 60 min at 4°C. The virus bands were collected, washed by dilution, centrifuged at 18,000 rpm (Beckman SW41 rotor) for 60 min at 4°C, resuspended in 0.2 ml of 0.1× TE, and stored at −20°C.

Fractionation of virions into the envelope and nucleocapsid. BV and ODV were fractionated into envelope and nucleocapsid preparations as previously described (7), with modifications. Approximately 200 μg of BV or ODV was incubated into 12 ml of 1.0% NP-40, 10 mM Tris, pH 8.5, at room temperature for 30 min with gentle agitation. The solution was centrifuged at 35,000 rpm (Beckman SW41 rotor) for 45 min at 4°C; the envelope proteins and nucleocapsid proteins were in the supernatant and pellet, respectively. (i) The envelope proteins in the supernatant were removed, precipitated with 4 volumes of acetone, and centrifuged at 35,000 rpm (Beckman SW41 rotor) for 45 min at 4°C, and the pelleted envelope proteins were dissolved in 10 mM Tris (pH 7.4). (ii) The pelleted nucleocapsids were washed by dilution, centrifuged at 35,000 rpm (Beckman SW41 rotor) for 45 min at 4°C, and resuspended in 10 mM Tris (pH 7.4).

Western blot analysis. Protein samples were mixed with equal volumes of 2× PSB and incubated at 95°C for 5 min. Samples were resolved by 12% SDS-

PAGE, transferred onto a nitrocellulose membrane (Schleicher & Schuel), and probed with one of the following primary antibodies: (i) rabbit polyclonal 38K antiserum (1:800) raised as described above, (ii) rabbit polyclonal VP39 antiserum (20) (1:1,000), or (iii) mouse monoclonal GP64 AcC6 antibody (1:3,000) (eBioscience). Alkaline phosphatase-conjugated goat anti-rabbit antibody (1:2,000) (Zymed Laboratories, Inc.) or alkaline phosphatase-conjugated goat anti-mouse antibody (1:2,000) (Zymed Laboratories, Inc.) were used as the secondary antibodies. The signals were detected with a BCIP (5-bromo-4-chloro-3-indolylphosphate)-Nitro Blue Tetrazolium kit (Roche Diagnostic Corporation). The apparent molecular mass of the bands was estimated by using Quantity One 1-D analysis software (Bio-Rad, Inc.).

Immunoelectron microscopy. Sf9 cells (5×10^6) were infected with wild-type AcMNPV at an MOI of 5 $TCID_{50}$ per cell. At 12, 24, 48, and 72 hpi, cells were dislodged and pelleted at 3,000 rpm for 5 min. For immunoelectron microscopy, cells were fixed in 2.5% (vol/vol) glutaraldehyde in PBS for 4 h at 4°C. Dehydration and embedding were performed as previously described (22). The procedures of sectioning, labeling, and observation were almost the same as those previously described (49). Ultrathin sections were obtained with a diamond knife and collected on Formvar-coated nickel grids. The ultrathin sections were floated on a drop of double-distilled water for 5 min, transferred to blocking solution (1% bovine serum albumin, 0.02% polyethylene glycol 20000, 100 mM NaCl, and 1% NaN_3) for 30 min, and incubated for 60 min with the 38K antiserum or the control glutathione *S*-transferase polyclonal antibodies at a dilution of 1:100 in blocking solution. After being washed with PBS for 5 min, the sections were incubated with 10-nm colloidal gold particles coated with protein A (Electron Microscopy Science, Inc.) at a dilution of 1:50 in blocking solution for 60 min, washed thoroughly with double-distilled water with several changes of water, air dried, and then stained for 20 s on drops of aqueous 1% uranyl acetate and for 20 s with 0.2% lead citrate. Samples were viewed with a JEM-100CX/II transmission electron microscope at an accelerating voltage of 80 kV.

Construction of the yeast two-hybrid clones. The ORFs of 38K and nine known nucleocapsid proteins, PP78/83, PCNA, VP1054, FP25, VLF-1, VP39, BV/ODV-C42, VP80, and P24, were cloned into both the GAL4 activation domain (AD) vector pGADT7 and the GAL4 DNA binding domain (BD) vector pGBKT7 (Clontech Laboratories, Inc.). To investigate if 38K interacts with other proteins that are not components of the nucleocapsid shell, the ORFs of the VS-associated protein PP31, the basic core protein P6.9, and the envelope protein of both BV and ODV, BV/ODV-E26, were also cloned into the yeast two-hybrid vectors. First, each ORF was amplified by PCR using the corresponding primer pairs (see Table S1 in the supplemental material) as follows: ac98528 and ac98324 for *38K*, ac95 and ac93 for *pp78/83*, ac495 and ac493 for *PCNA*, ac545 and ac543 for *vp1054*, ac615 and ac613 for *fp25*, ac775 and ac773 for *vlf-1*, ac895 and ac893 for *vp39*, ac1015 and ac1013 for *bv/odv-c42*, ac1045 and ac1043 for *vp80*, ac1295 and ac1293 for *p24*, ac365 and ac363 for *pp31*, ac1005 and ac1003 for *p6.9*, and ac165 and ac163 for *bv/odv-e26*. All PCR products were first cloned into the pMD 18-T vector to generate recombinant plasmids T-38K, T-PP78/83, T-PCNA, T-VP1054, T-FP25, T-VLF1, T-VP39, T-C42, T-VP80, T-P24, T-PP31, T-P6.9, and T-E26 for sequencing before being cut with the designated enzymes and subcloned into pGADT7 to generate pAD-38K and pAD-PP78/83, etc., respectively. These clones are designated pAD-X, where X represents an individual gene.

Second, all 13 ORFs were cloned into pGBKT7, and the resultant clones are designated pBD-X clones, where X represents an individual gene. For *38K*, *vlf-1*, *vp39*, *pp31*, *p6.9*, and *bv/odv-e26*, the ORFs were released with EcoRI and BamHI from T-38K, T-VLF1, T-VP39, T-PP31, T-P6.9, and T-E26, respectively, and subcloned into pGBKT7 to generate pBD-38K, pBD-VLF1, pBD-VP39, pBD-PP31, pBD-P6.9, and pBD-E26. For *pp78/83*, *fp25*, *bv/odv-c42*, and *p24*, the ORFs were released with EcoRI and PstI from T-PP78/83, T-FP25, T-C42, and T-P24, respectively, and subcloned into pGBKT7 to generate pBD-PP78/83, pBD-FP25, pBD-C42, and pBD-P24. The *vp80* ORF was released with BamHI and PstI from T-VP80 and subcloned into pGBKT7 to generate pBD-VP80. Primers ac495 and ac4933 (see Table S1 in the supplemental material) were used to amplify *PCNA* by PCR, and the product was cut with BamHI and SalI before being subcloned into pGBKT7 to generate pBD-PCNA. Primers ac545 and ac5432 (see Table S1 in the supplemental material) were used to amplify *vp1054* by PCR, and the product was cut with BamHI and PstI before being subcloned into pGBKT7 to generate pBD-VP1054.

Yeast two-hybrid interaction testing. Pairs of AD and BD fusion plasmids were cotransformed into the AH109 *Saccharomyces cerevisiae* strain (*MAT***a** trp1-901 leu2-3 112 ura3-52 his3-200 *gal4*Δ *gal80*Δ *LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3* $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2 URA3*::$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ MEL1*) according to the manufacturer's instructions (Clontech Laboratories, Inc.), with modifications. Transformants were first plated onto synthetic complete medium (SCM) plates lacking tryptophan and leucine (SCM-2). The plates were incubated for 3 days at 30°C, and the interactions were assessed by streaking the transformants onto SCM-2 (control), SCM-3 (lacking tryptophan, leucine, and histidine), and SCM-4 (lacking tryptophan, leucine, histidine, and adenine) plates. The plates were incubated for 3 to 4 days at 30°C, and interactions were considered strong when colonies formed on all three types of plates and weak when colonies formed on SCM-3 as well as SCM-2 plates but not on SCM-4 plates.

FLAG- and HA-tagged constructs. Four primer pairs were designed to construct FLAG- and hemagglutinin (HA)-tagged expression plasmids. The ORFs were amplified by PCR using primer pairs as follows: ac5452 and ac5433 for *vp1054*, ac8952 and ac8932 for *vp39*, ac98529 and ac98326 for *38K*, and ac10452 and ac10432 for *vp80*. The PCR products were first cloned into the pMD 18-T vector for sequencing before being subcloned into pHSFLAGHisVi+ (kindly provided by A. Lorena Passarelli, Kansas State University) (8) to generate the FLAG-tagged constructs pFLAG-VP1054, pFLAG-VP39, pFLAG-38K, and pFLAG-VP80, respectively. To generate the HA-tagged 38K expression plasmid, the 38K PCR product was subcloned into pHSEpiHisVI+ (kindly provided by A. L. Passarelli) (8) to give pHA-38K.

Double transfection and co-IP assays. To confirm the interactions detected by yeast two-hybrid assays, co-IP assays were performed as previously described (51), with some modifications. Sf9 cells (5×10^6 plated on 25-cm^2 cell culture flasks) were cotransfected with 2.5 μg of pHA-38K and 2.5 μg of pFLAG-VP1054, pFLAG-VP39, pFLAG-38K, pFLAG-VP80, or pHSFLAGHisVi+ plasmid, using 18 μl of Cellfectin liposome reagent (Invitrogen Life Technologies). The cells were incubated for 5 h after addition of the transfection mixture, washed twice with TNM-FH medium, replenished with 2 ml of fresh TNM-FH medium supplemented with 10% fetal bovine serum, and incubated for 12 h at 27°C. To increase the transfection efficiency, a second cotransfection was performed 12 h after the first cotransfection. The cells were then incubated in fresh medium for 24 h at 27°C, heat shocked for 30 min at 42°C, and then treated for 30 min at 27°C with 50 μg/ml Z-Leu-Leu-Leu-Leu-Al (MG 132; Sigma-Aldrich Co.), a proteasome inhibitor (8). After 4 to 6 h, cells were lysed with 500 μl of lysis/immunoprecipitation buffer [as described in reference 51, except that $(NH_4)_2SO_4$ was changed from 150 to 100 mM]. The lysate was incubated for 15 min at 4°C with mixing and then centrifuged at 14,000 rpm to clarify the lysate. The extract was precleared by adding 50 μl of a 50% slurry of protein G beads (Zymed Laboratories, Inc.), followed by incubation at 4°C for 1 h with rolling. The supernatant was transferred to a fresh 1.5-ml Eppendorf tube and mixed with 7.5 μg of anti-FLAG antibody (Sigma-Aldrich Co.) or the control mouse immunoglobulin G (IgG; Pierce). After incubation at 4°C for 1 h with rolling, 50 μl of a 50% slurry of protein G beads was added to the mixture, followed by incubation at 4°C for 1 h with rolling. The beads were collected by centrifugation at 4,000 rpm for 2 min at 4°C, washed four times with 1 ml of lysis/immunoprecipitation buffer [100 mM $(NH_4)_2SO_4$] for 10 to 15 min each time, and boiled in 25 μl of 2× PSB for 5 min. The dissolved immunoprecipitates (10 μl/lane) as well as the input cell lysates (10 μl/lane after 1:1 dissolution in 2× PSB) were resolved by 10% SDS-PAGE; transferred to a nitrocellulose membrane (Schleicher & Schuel) for Western blotting with anti-FLAG (1 μg/ml), anti-HA (1 μg/ml), and anti-38K (1:500) antibodies separately; and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:20,000). The blots were developed by use of SuperSignal West Pico chemiluminescent substrate (Pierce) and exposure to X-ray films.

RESULTS

Temporal expression of 38K during infection. To obtain polyclonal rabbit antibodies against 38K, 38K was expressed in *E. coli* as a His-38K fusion protein and purified by chromatography on a Ni-chelating affinity column before immunization of rabbits to obtain the 38K antiserum. The time course of 38K expression in AcMNPV-infected Sf9 cells was analyzed by Western blotting using the 38K antiserum. A major immunoreactive band of approximately 38 kDa was first detected at 18 hpi, and it persisted up to 96 hpi (Fig. 1). From 48 hpi to 96 hpi, two smaller immunoreactive bands of approximately 29 kDa and 26 kDa were detected (Fig. 1). It is possible that 38K might be degraded or posttranslationally modified at late infection, or the 38K antibodies raised in this study may cross-react to the

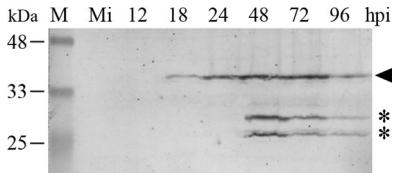

FIG. 1. Time course of 38K synthesis in AcMNPV-infected *S. frugiperda* cells. Sf9 cells were infected or mock-infected with AcMNPV at an MOI of 5. At different time points postinfection as indicated, cells were harvested, and total cellular proteins were resolved on 12% SDS-PAGE and analyzed by Western blotting. The blot was probed with the 38K polyclonal antiserum. Mi, mock infection Sf9 cells; M, prestained protein marker. The numbers at the left indicate the molecular sizes (in kilodaltons) of the protein standards. The arrowhead indicates the expected bands of 38K detectable at 18 hpi, and the asterisks indicate two smaller immunoreactive bands from 48 hpi.

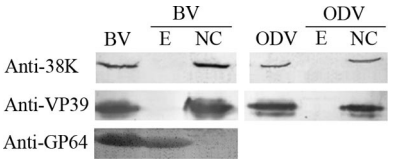

FIG. 2. Localization of 38K in purified and fractionated virions. BV and ODV were purified by sucrose gradient and fractionated into envelope and nucleocapsid preparations. Samples were resolved on 12% SDS-PAGE gels and analyzed by Western blotting. The blots were probed with the 38K polyclonal antiserum to detect the localization of 38K to the viral structure, with anti-VP39 to detect the nucleocapsid protein VP39, and with anti-GP64 to detect the BV envelope protein GP64. BV, purified BV; ODV, purified ODV; E, envelope; NC, nucleocapsid.

29-kDa and 26-kDa unidentified proteins. To assess the specificity of the rabbit polyclonal 38K antiserum, Western blotting was carried out using total protein extracts of Sf9 cells transfected with a 38K knockout AcMNPV mutant ($vAc^{38K\text{-}KO\text{-}PH\text{-}GFP}$) (49) at different time points posttransfection. No signal was observed after incubation with the rabbit polyclonal 38K antiserum (see Fig. S1 in the supplemental material).

38K is associated with the nucleocapsids of BV and ODV. Since 38K is required for nucleocapsid assembly, it is possible that 38K is required for regulation of nucleocapsid assembly or is a structural component that is essential for nucleocapsid maturation. We first determined if 38K was associated with the baculovirus virions. BV and ODV were prepared from AcMNPV-infected larva, purified, and then analyzed by Western blotting with the 38K antiserum. As shown in Fig. 2, the 38K antibodies reacted strongly with only one peptide, with a molecular mass of 38 kDa, in BV and ODV samples, indicating that 38K was associated with BV and ODV. To identify the location of 38K more exactly in the virus structure, BV and ODV were biochemically fractionated into the nucleocapsid and envelope fractions (Fig. 2) for Western blotting, and 38K was detected in the nucleocapsid but not the envelope fractions of BV and ODV. As controls with which to confirm the efficiency of the fractionation, the nucleocapsid protein VP39 and the BV envelope protein GP64 were also analyzed by Western blotting, and both VP39 and GP64 were detected in the expected nucleocapsid and envelope fractions, respectively (Fig. 2). These results indicated that 38K is associated with both the BV and the ODV virions and localized to the nucleocapsids of BV and ODV.

Immunoelectron microscopic analysis of 38K localization to the virus structure. To further investigate if 38K is associated with any specialized structure in AcMNPV-infected Sf9 cells, immunoelectron microscopy was carried out. The virus-infected cells could be easily distinguished from the mock-infected cells (Fig. 3A) by the presence of the VS, which is an electron-dense, baculovirus-induced structure within the nucleus (Fig. 3B). Maturation of the VS yielded a significant and morphologically distinct peristromal compartment of the nucleoplasm, called the ring zone (Fig. 3B). The nucleocapsids align with the vesicle-like structures of de novo envelopes, and bundles of the nucleocapsids prior to occlusion in the protein-crystalline matrix of the developing occlusion bodies are often observed within this region (45). At 24 hpi, gold particles appeared to be distributed over the cylindrical capsid sheath within the electron-lucent intrastromal spaces, while few, if any, gold particles appeared within the electron-dense matte (Fig. 3C). Gold particles appeared to be distributed over the cylindrical capsid sheath of nucleocapsids of the mature and premature ODVs within the ring zone (Fig. 3C). At 48 hpi, the nucleocapsids aligned with the bundle formation or enveloped in the mature ODV within the ring zone were stained by the gold particles (Fig. 3D and E). Within the polyhedra, gold particles appeared to be specifically localized to the virions and did not stain other regions of the polyhedra (Fig. 3F). At 72 hpi, gold particles were found on the nucleocapsids of the ODV that were embedded in the polyhedra, and the nucleocapsids in the VS could no longer be seen as the VS shrank (data not shown). Gold particles were not distributed specifically to the bases or the nipple caps of the nucleocapsids, suggesting that 38K is not a component of these specialized structures (Fig. 3C to F). Although the VS could be detected in some of the cells at 12 hpi, no gold particle was observed, and no nucleocapsid shell was present at this time point (data not shown). The 38K antiserum did not cross-react with any component in uninfected cells (data no shown). Preimmune serum and glutathione *S*-transferase polyclonal antibodies were used as controls, and immunogold staining was not detected in the infected cells (data not shown). These results demonstrated that 38K is related to the nucleocapsid, suggesting that 38K is a capsid structural protein.

Interaction of 38K with nucleocapsid proteins as determined by yeast two-hybrid assays. The results described above indicated that 38K is a structure component of the nucleocapsids of BV and ODV. To further illustrate the relationship between 38K and the nucleocapsid, yeast two-hybrid assays were used to determine whether 38K physically interacts with other known nucleocapsid proteins. 38K and nine previously known nucleocapsid proteins, PP78/83, PCNA, VP1054, FP25, VLF-1, VP39, BV/ODV-C42, VP80, and P24, were selected for testing. The potential interactions of 38K with the VS-associated protein PP31, the DNA binding protein P6.9, and the envelope protein of both BV and ODV, BV/ODV-E26, were also tested to determine if 38K is specifically associated with nucleocapsid proteins. The ORFs of the 13 genes were individually cloned into the GAL4 AD vector pGADT7 to generate the AD fusion constructs designated pAD-X and into

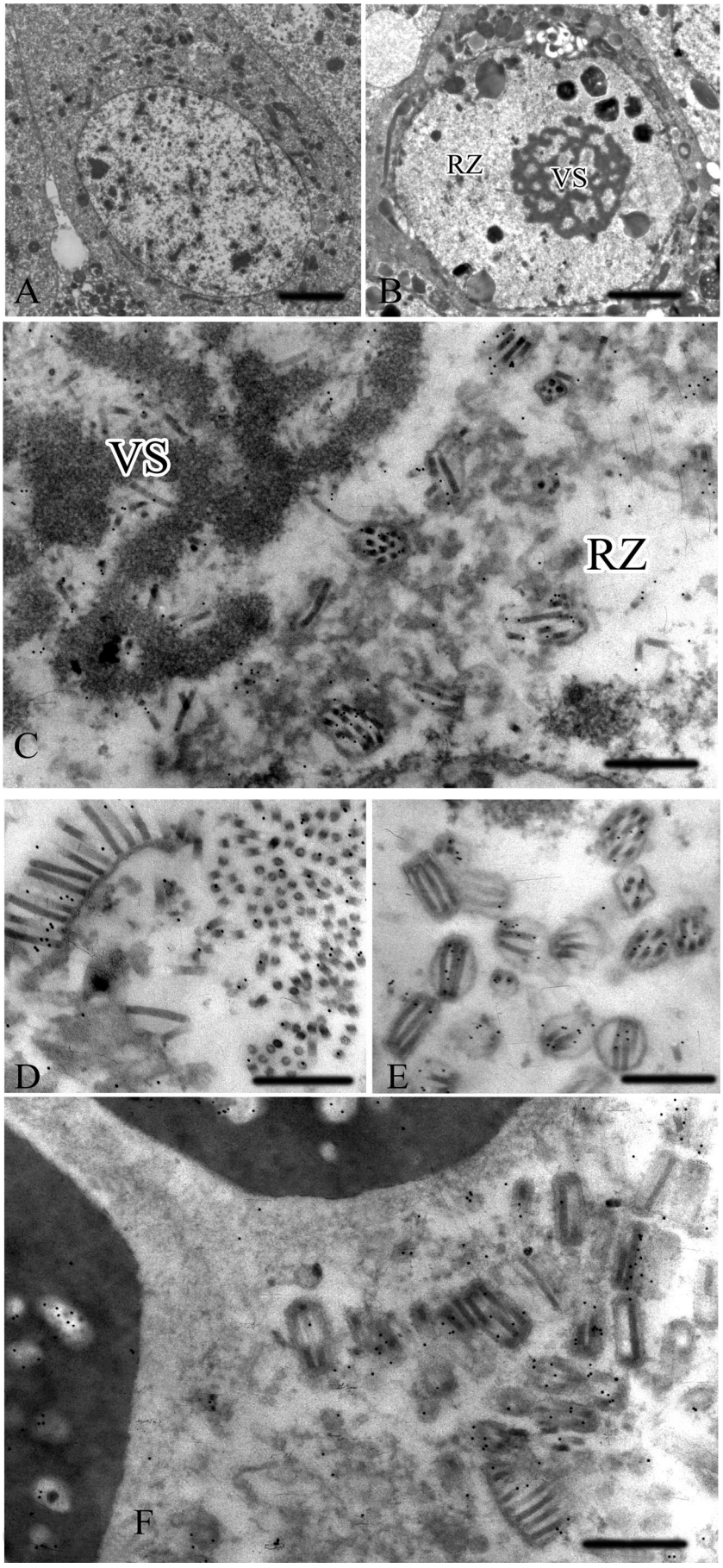
A
B
RZ
VS
C
VS
RZ
D
E
F

TABLE 1. Yeast two-hybrid tests of interaction of BD-38K with other proteins[a]

AD construct	Growth level for indicated plasmid and plate					
	SCM-2		SCM-3		SCM-4	
	pGBKT7	pBD-38K	pGBKT7	pBD-38K	pGBKT7	pBD-38K
pGADT7	+++	+++	−	+/−	−	−
pAD-38K	+++	+++	−	+++	−	++
pAD-PP78/83	+++	+++	−	+/−	−	−
pAD-PCNA	+++	+++	−	+/−	−	−
pAD-VP1054	+++	+++	−	+++	−	++
pAD-FP25	+++	+++	−	+/−	−	−
pAD-VLF1	+++	+++	−	+/−	−	−
pAD-VP39	+++	+++	−	+++	−	++
pAD-C42	+++	+++	−	+/−	−	−
pAD-VP80	+++	+++	−	++	−	−
pAD-P24	+++	+++	−	+/−	−	−
pAD-PP31	+++	+++	−	+/−	−	−
pAD-P6.9	+++	+++	−	+/−	−	−
pAD-E26	+++	+++	−	+/−	−	−

[a] Each GAL4 AD fusion construct was cotransformed with the GAL4 BD fusion construct pBD-38K or the empty GAL4 BD plasmid pGBKT7. Levels of colony growth on plates are as follows: −, no growth; +/−, minimum (background) growth; +, weak growth; ++, intermediate growth; +++, strong growth.

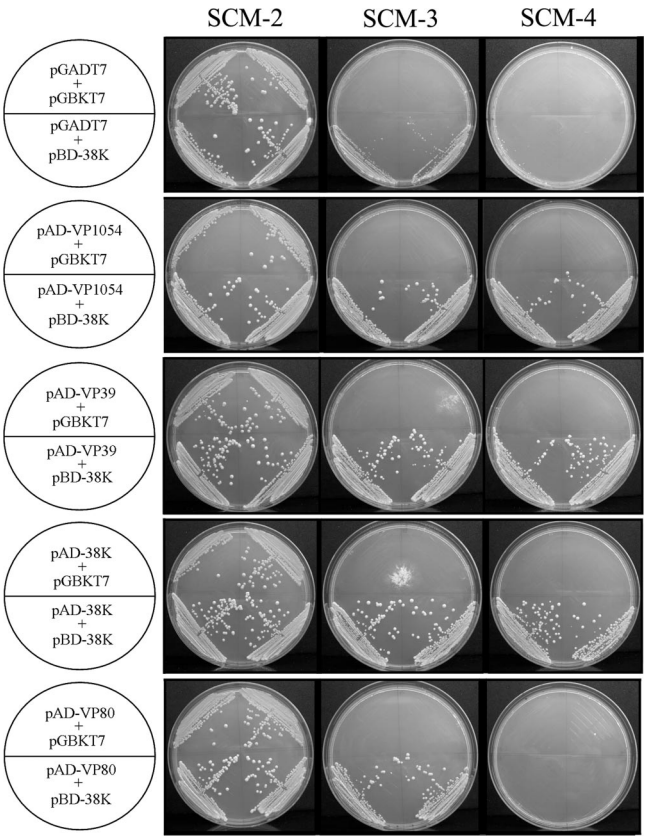

FIG. 4. Yeast two-hybrid tests of interactions of 38K with other proteins. The AD fusion plasmid and the BD fusion plasmid were cotransformed into AH109 yeast cells. Transformants were plated on SCM plates lacking tryptophan and leucine (SCM-2) and grown for 3 days at 30°C. The potential interactions were assessed by transferring colonies to SCM plates lacking tryptophan, leucine, and histidine (SCM-3) and SCM plates lacking tryptophan, leucine, histidine, and adenine (SCM-4). The plates were incubated for 3 to 4 days at 30°C. The names of the cotransformed plasmids and the plates are indicated.

the GAL4 BD vector pGBKT7 to generate the BD fusion constructs designated pBD-X, where X represents an individual protein of interest.

For interaction testing in one direction (pBD-38K/pAD-X), each of the AD fusion constructs was cotransformed with pBD-38K, and for the other direction (pBD-X/pAD-38K), each of the BD fusion constructs was cotransformed with pAD-38K for analysis. Cotransformations of each pAD-X plasmid with the empty vector pGBKT7, of each pBD-X plasmid with the empty vector pGADT7, and of pGBKT7 with pGADT7 were used as the negative controls. Cotransformation of pGBKT7-p53 and pGADT7-T-antigen was used as the positive control (data not shown). Transformants were first plated onto SCM-2 agar plates, and the colonies were streaked onto SCM-2, SCM-3, and SCM-4 plates. The interactions were assessed by growth on SCM-3 and/or SCM-4 plates, on which interaction between the candidate proteins is necessary for cell growth and colony formation. Interactions were considered strong if colonies formed on all three types of plates and weak if colonies were observed on SCM-3 as well as SCM-2 plates but not SCM-4 plates. The results of the testing are shown in Table 1 and Fig. 4. In comparison to the control levels, strong interactions were detected between BD-38K and AD-VP1054, AD-VP39, and AD-38K, and a weak interaction was observed between BD-38K and AD-VP80 (Table 1 and Fig. 4). A very weak interaction was also detected between AD-38K and BD-VLF1 (Table 2). All fusion proteins in both vectors were expressed in yeast cells as determined by Western blotting, except that AD-PP78/83 and AD-FP25 were barely detectable and AD-PCNA could not be examined, as it would comigrate with a cross-reactive band by the anti-HA antibody (data not shown).

Confirmation of the interactions by co-IP assays. To confirm the interactions between 38K and VP1054, VP39, VP80, and 38K itself detected by the yeast two-hybrid assays, co-IP assays were performed using extracts from Sf9 cells cotransfected with pHA-38K and pFLAG-VP1054, pFLAG-VP39, pFLAG-38K, pFLAG-VP80, or the control vector pHSFLAGHisVI+. Cells were harvested at 24 h posttransfection and lysed for immunoprecipitation. Lysates were immunoprecipitated with anti-FLAG antibody or the control mouse IgG. The lysates and immunoprecipitates were analyzed by

FIG. 3. Immunoelectron microscopy of uninfected and baculovirus-infected Sf9 cells, using the 38K antiserum. (A) Cross-section of uninfected Sf9 cells. (B) Cross-section of infected Sf9 cells harvested at 48 hpi. (C) Portion of the nucleus of an infected Sf9 cell at 24 hpi. Nucleocapsids in the VS and ring zone (RZ) were stained with the gold particles. (D to F) Portion of the nucleus of an infected Sf9 cell at 48 hpi. (D) Staining of bundled nucleocapsids in the ring zone of the nucleus. (E) Staining of mature ODV in the ring zone of the nucleus. (F) Staining of polyhedra and premature or mature ODV. The bars represent 5.0 μm (A and B) or 0.5 μm (C to F).

TABLE 2. Yeast two-hybrid tests of interaction of AD-38K with other proteins[a]

BD construct	Growth level for indicated plasmid and plate					
	SCM-2		SCM-3		SCM-4	
	pGADT7	pAD-38K	pGADT7	pAD-38K	pGADT7	pAD-38K
pGBKT7	+++	+++	−	−	−	−
pBD-38K	+++	+++	−	+++	−	++
pBD-PP78/83	+++	+++	−	−	−	−
pBD-PCNA	+++	+++	−	−	−	−
pBD-VP1054	+++	+++	−	−	−	−
pBD-FP25	+++	+++	−	−	−	−
pBD-VLF1	+++	+++	−	+	−	−
pBD-VP39	+++	+++	−	−	−	−
pBD-C42	+++	+++	−	−	−	−
pBD-VP80	+++	+++	−	−	−	−
pBD-P24	+++	+++	−	−	−	−
pBD-PP31	+++	+++	−	−	−	−
pBD-P6.9	+++	+++	−	−	−	−
pBD-E26	+++	+++	−	−	−	−

[a] Each GAL4 BD fusion construct was cotransformed with the GAL4 AD fusion construct pAD-38K or the empty GAL4 AD plasmid pGADT7. Colonies that grew on SCM-2 plates were streaked on SCM-2, SCM-3, and SCM-4 plates. Levels of colony growth on plates are as follows: −, no growth; +/−, minimum (background) growth; +, weak growth; ++, intermediate growth; +++, strong growth.

Western blotting with anti-FLAG antibody to detect the expression and immunoprecipitation of the FLAG-tagged proteins and anti-38K and anti-HA antibodies to determine if HA-38K was coimmunoprecipitated with the FLAG-tagged proteins. As shown in Fig. 5, all of the FLAG-tagged proteins could be detected by the anti-FLAG antibody in the input lysates and anti-FLAG immunoprecipitates, and HA-38K was clearly detectable by the 38K antiserum or anti-HA antibody in the immunoprecipitates of FLAG-tagged VP1054, VP39, and 38K (Fig. 5), which have strong interactions with 38K in the yeast two-hybrid assays. Note that 38K antiserum reacted with FLAG-38K as well as HA-38K, resulting in a strong signal in the anti-FLAG immunoprecipitate from the lysate expressing both FLAG-38K and HA-38K. HA-38K was barely detectable by the anti-HA antibody in the FLAG-VP80 immunoprecipitate (Fig. 5), consistent with their interaction being weak in the yeast two-hybrid system. No immunoblotting signal was observed in the vector or mouse IgG controls for any pair (Fig. 5). These co-IP data are consistent with results from the yeast two-hybrid assays and suggest that 38K interacts strongly with VP1054, VP39, and 38K and weakly with VP80 in vivo.

DISCUSSION

Recently, we showed that 38K is essential for AcMNPV replication, as the deletion of a part of the 38K coding region caused a failure of the viral nucleocapsid assembly (49). It is not known whether the phenotype of the 38K-null mutant is the result of a lack of a structural protein involved directly in the process of nucleocapsid assembly or of pleiotropic effects. A recent study revealed that CuniNPV ORF87, a homolog of AcMNPV 38K, is a component of the CuniNPV ODV (33), which impelled us to investigate the relationship between AcMNPV 38K and the virion. In this report, we demonstrated that 38K is a baculovirus nucleocapsid structural protein and interacts with other nucleocapsid proteins, including VP1054, VP39, VP80, and 38K itself.

We first raised polyclonal antibodies to the His-38K fusion protein and determined the expression time course of 38K in AcMNPV-infected Sf9 cells. The antibodies identified a specific 38-kDa polypeptide from the lysates of virus-infected cells at time points from 18 hpi to 96 hpi. The apparent molecular mass of 38K encoded by AcMNPV 38K was determined for the first time in the present study and is consistent with the predicted molecular mass (37,978 Da) of AcMNPV 38K (23). During infection, baculoviral genes are expressed in a coordinated fashion: early genes are expressed prior to the initiation of viral DNA replication, which occurs at about 6 hpi in AcMNPV-infected Sf9 cells, whereas genes expressed concomitantly with viral DNA replication are classified as late genes (41, 45). Early genes appear to be involved in the regulation

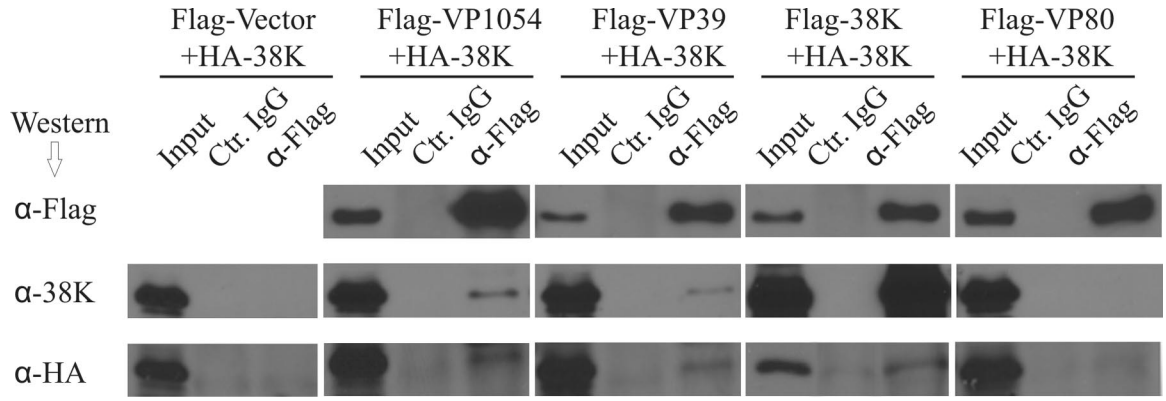

FIG. 5. Co-IP assays used to confirm interactions of 38K with VP1054, VP39, VP80, and 38K itself. Sf9 cells were cotransfected twice with pHA-38K and individual plasmids containing FLAG-tagged ORFs of interest or the pHSFLAGHisVi+ plasmid. At 24 h posttransfection, cells were heat shocked to induce the expression of the transfected genes, collected, and lysed for immunoprecipitation with anti-FLAG antibody. Western blots were probed with anti-FLAG antibody to show the expression and immunoprecipitation of the FLAG-tagged proteins and with anti-38K and anti-HA antibodies, separately, to detect the expression and co-IP of HA-38K. Input, input cell lysates; Ctr. IgG, immunoprecipitation with control mouse IgG; α-FLAG, immunoprecipitation with anti-FLAG antibody.

and expression of other viral genes, but many late genes encode structural proteins for the virion (28). A previous study showed that *38K* is transcribed at late times postinfection and that its transcription initiates from the late promoter start site consensus sequence TAAG (24). In the present study, the temporal expression pattern of 38K is characteristic of a baculovirus late protein, implying that 38K might be a structural protein. In a previous study, Braunagel et al. performed proteomic analysis using multiple approaches to identify proteins associated with the AcMNPV ODV; however, 38K was not identified to be a component of ODV or BV (6). Four other known nucleocapsid proteins, PCNA, VP1054, P24, and BV/ODV-E26, were not detected either in their study. This may be due to the masking of the low-abundance proteins by the highly expressed proteins. In this study, the 38K antibodies provided us with a more specific and sensitive tool for detecting the location of 38K protein in the viral structure. Subsequent investigation on the relationship between 38K and the virions by Western blotting revealed that 38K is associated with both BV and ODV and is a component of the nucleocapsid, not of the envelope. Western blotting revealed two other, smaller bands, of approximately 29 kDa and 26 kDa, by 48 hpi in the virus-infected Sf9 cells. This may be due to proteolytic cleavage or other modification of 38K by protein enzymes or nonspecific cross-reactive bands. These smaller peptides were not found to be associated with the nucleocapsids of ODV and BV (data not shown).

To further substantiate that 38K is a nucleocapsid protein, we performed immunoelectron microscopic analysis and identified the intranuclear distribution of 38K. This is consistent with our previous results indicating that green fluorescent protein-fused 38K expressed from a recombinant virus localized to the center of the nucleus (49). Furthermore, this study showed that 38K was specifically detected in the nucleocapsids within the intrastromal space and in the nucleocapsids of the bundle formations, the ODVs, and the mature polyhedra within the ring zone. In contrast, the areas surrounding the nucleocapsids, such as the electron-condensed nucleic acid region of the stroma, the membrane profiles of the enveloping nucleocapsid bundles or of ODVs, or the protein matrix of polyhedra, showed no obvious 38K staining. Gold particles appeared to be distributed over the cylindrical capsid sheath of nucleocapsids, which is similar to the localization pattern of the major capsid shell protein VP39 (37). The localization pattern of 38K is different from that of PP78/83, which is associated with the ends of the capsids containing the basal structure (36); from that of PP31, which is associated with the electron-condensed matte (14); and from that of ODV-E66, which is associated with the intranuclear microvesicles and the envelope of ODV (17). So the distribution of 38K suggests that it may be a capsid shell protein associated with VP39 and other nucleocapsid proteins.

To investigate the relationship between 38K and baculovirus nucleocapsid proteins in more detail, yeast two-hybrid assays were performed. Ten nucleocapsid proteins, 38K, PP78/83, PCNA, VP1054, FP25, VLF-1, VP39, BV/ODV-C42, VP80, and P24, were tested for potential interactions with 38K. The possible interactions of 38K with the VS-associated protein PP31, the DNA binding protein P6.9, and the envelope protein of both BV and ODV, BV/ODV-E26, were also examined to determine if 38K specifically associates with the nucleocapsid. The results of yeast two-hybrid assays revealed that three nucleocapsid proteins, VP39, VP1054, and 38K itself, showed strong interactions with 38K, and VP80 showed a weak interaction with 38K. The strong interactions were confirmed by co-IP assays using insect cell lysates expressing the interacting proteins. VP39, with its abundance and distribution throughout the nucleocapsid, is the major capsid protein that builds up the skeleton of the nucleocapsid (37, 40). VP1054 is required for nucleocapsid formation, and a certain single-amino-acid alteration of it leads to the failure of normal nucleocapsid formation (31). VP80 was shown to be a nucleocapsid component of both BV and ODV which is expressed late in infection and concentrated in infected cell nuclei (30). We found that 38K interacts with itself, confirming a previous study indicating that 38K proteins interact with each other as homo-oligomers (15). The characterization of 38K as a nucleocapsid structural protein and the interactions detected between 38K and other nucleocapsid proteins provide more information with which to study the structure of the baculovirus nucleocapsid and the functions of some critical structural proteins and also facilitate comparative studies of AcMNPV with other baculoviruses. Recently, two new nucleocapsid proteins, EXON0 and AC142, were identified. EXON0 was shown to be a structural protein of both BV and ODV found in the nucleocapsid fraction of BV by Western blotting, and EXON0 interacts with BV/ODV-C42 and FP25 (10, 11). The AC142 gene, one of the 29 core genes among all sequenced baculoviruses to date, was also found to encode a nucleocapsid structural protein (26). The interactions between 38K and these two proteins were not investigated in the current study and need further determination.

Our previous studies indicated that 38K does not affect the replication of viral DNA, and the masses of electron-lucent tubular structures containing the capsid protein VP39 are present in cells transfected with 38K knockout virus (49). Thus, the structural protein 38K is not necessary for capsid shell formation; however, our results suggest that 38K plays an important role in facilitating viral genome packaging into the empty capsid shell. Virus assembly is a complex process that requires the temporal and coordinated activities of numerous proteins. The interactions of 38K with VP39, VP1054, and VP80, as well as 38K itself, identified in this study imply that these proteins may be jointly involved in a crucial step in viral DNA entry into the capsid during packaging. However, understanding of the details of this process requires further study.

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